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A *Bacteroides ovatus* Chromosomal Locus Which Contains an α-Galactosidase Gene May Be Important for Colonization of the Gastrointestinal Tract

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An α-galactosidase gene has been cloned from the human colonic *Bacteroides* species *Bacteroides ovatus* 0038. This α-galactosidase appears to be distinct from two previously characterized α-galactosidases, I and II, from the same strain and has been designated α-galactosidase III. Partially purified α-galactosidase III from *Escherichia coli* EM24 containing pFG61ΔSE had a pI of 7.6, as compared with the reported pI values for the known α-galactosidases of 5.6 for I and 6.9 for II. Its molecular weight as estimated on sodium dodecyl sulfate-polyacrylamide gels was 78,000, whereas the molecular weights of α-galactosidases I and II were 85,000 and 80,500, respectively. The only substrate hydrolyzed by α-galactosidase III was melibiose, whereas the other two α-galactosidases were able to degrade melibiose, raffinose, and stachyose and partially degraded guar gum. α-Galactosidase III had a pH optimum of 6.7 to 7.2. Finally, a single crossover insertion which disrupted the gene in the *B. ovatus* chromosome had no effect on expression of α-galactosidases I and II. Although this insertion had no effect on the ability of *B. ovatus* to grow in laboratory medium on any of the galactoside-containing carbohydrates tested, the insertion mutant was outcompeted by wild type when a combination of mutant and wild type was used to colonize germfree mice. Insertions on either side of the gene had the same effect. Thus, the locus which contains α-galactosidase III may be important for colonization in vivo. Unexpectedly, when the galactoside-containing carbohydrates were tested for induction of α-galactosidase III, two additional α-galactosidases were detected, bringing the total number of α-galactosidases produced by *B. ovatus* to five.

Bacteroides is a genus of gram-negative obligately anaerobic bacteria which accounts for approximately 20% of the bacteria found in the human colon (7, 13, 17). *Bacteroides*, like most other colonic anaerobes, requires a fermentable carbohydrate for growth (8). However, colonic *Bacteroides* spp. differ from the other colonic anaerobes in their ability to utilize a wide range of complex polysaccharides (15, 18). Since most of the carbohydrate that reaches the colon is in the form of polysaccharides (15), the ability of *Bacteroides* species to utilize polysaccharides probably contributes to the predominance of *Bacteroides* in the colonic microflora.

Most of the previous work on polysaccharide utilization by *Bacteroides* has focused on linear polysaccharides such as chondroitin sulfate, amylose, or polygalacturonic acid (17). However, many of the polysaccharides that enter the colon are complex branched polysaccharides. Since many of these branched polysaccharides contain some proportion of α-galactoside residues, we have been investigating α-galactoside utilization by *Bacteroides*. In these studies, we have used *Bacteroides ovatus* because it is the colonic species which ferments the greatest variety of branched polysaccharides (18).

Previously, Gherardini et al. (4) purified and characterized two α-galactosidases from *B. ovatus* 0038. The first enzyme, designated α-galactosidase I, was expressed when *B. ovatus* was grown on the branched galactomannan guar gum. Guar gum consists of a β(1-4)-linked mannose backbone with α(1-6)-linked galactose residues (4). The second enzyme, designated α-galactosidase II, was expressed when *B. ovatus* was grown on simple α-galactosides such as melibiose, raffinose, and stachyose. Both α-galactosidases I and II were able to remove galactose residues from melibiose, raffinose, and stachyose and partially degraded guar gum.

Our original goal had been to clone one or both of the *B. ovatus* α-galactosidase genes to study their expression and function in *B. ovatus*. By screening a cosmid library of *B. ovatus* DNA, we found one clone that expressed α-galactosidase activity in *Escherichia coli*. However, this gene has proven to be a third α-galactosidase which is distinct from α-galactosidases I and II. In the present paper we describe the characterization of this new α-galactosidase. We also present evidence that the chromosomal locus which contains the cloned α-galactosidase may be ecologically significant. Finally, we demonstrate that *B. ovatus* produces two additional α-galactosidases, one of which has been found only in cells grown on porcine gastric mucin or gum tragacanth.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Growth conditions. *E. coli* strains were grown in Luria broth or agar (LB or LA). *Bacteroides* strains were grown in prereduced Trypticase (BBL Microbiology systems, Cockeysville, Md.)-yeast extract-glucose (TYG) broth or agar or in a defined basal medium similar to that developed by Varel and Bryant and modified as described previously (22). In both cases, the atmosphere was CO_2 (20%)–N_2 (80%). Stock cultures of *Bacteroides* strains were maintained at room temperature in chopped-meat broth (8). Defined medium containing guar gum as the sole carbohydrate source was prepared as described previously (4). Defined medium containing larch arabinogalactan, polygalacturonic acid, pectin, xylan, gum tragacanth, porcine gastric mucin, or trypsin inhibitor as the sole carbohydrate was made similarly to the

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant phenotype[a]	Source
E. coli		
EM24	RecA$^-$ Str^r	RecA$^-$ derivative of LE392 (12)
M2508	*melA7*	CGSC 4926 (20)
B. ovatus		
0038	Em^s Gen^r	Type strain (Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg)
AR	Em^r Gen^r	Contains pVAL-AR inserted in the chromosome of *B. ovatus* 0038 (this study)
AH	Em^r Gen^r	Contains pVAL-AH inserted in the chromosome of *B. ovatus* 0038 (this study)
RR	Em^r Gen^r	Contains pVAL-RR inserted in the chromosome of *B. ovatus* 0038 (this study)
Plasmids		
pBR328	Tc^r Ap^r Cm^r	2
pNJR-5	Kn^r (Mob$^+$ Rep$^+$) (Em^r)	21
pNJR-6	Kn^r (Mob$^+$ Rep$^-$) (Em^r)	23
pHC79	Ap^r Cos site	6
p27-2	Ap^r Cos site	pHC79 containing 40-kb insert encoding α-galactosidase activity (this study)
pFG61	Tc^r Ap^r	Contains initial 10-kb *Eco*RI subclone from p27-2 in the *Eco*RI site of pBR328 (this study)
pFG61ΔS	Ap^r	pFG61 digested with *Sph*I and religated (this study)
pFG61ΔSE	Ap^r	pFG61ΔS digested with *Eco*RI and religated (this study)
pFG61ΔSEA	Ap^r	pFG61ΔSE digested with *Ava*I and religated (this study)
pVAL200	Kn^r (Em^r)	pNJR-5 containing the 4.5-kb *Bam*HI fragment cloned into the *Bam*HI site (this study)
pVAL250	Kn^r (Em^r)	pNJR-5 containing the 8.8-kb *Eco*RI fragment cloned into the *Eco*RI site (this study)
pVAL260	Ap^r Cm^r	pBR328 containing the 3.7-kb *Hin*dIII fragment cloned into the *Hin*dIII site (this study)
pVALRR	Kn^r (Mob$^+$ Rep$^-$) (Em^r)	pNJR-6 containing the 0.6-kb *Eco*RI fragment cloned into the *Hpa*I site (this study)
pVALAR	Kn^r (Mob$^+$ Rep$^-$) (Em^r)	pNJR-6 containing the 1.6-kb *Ava*I-*Eco*RV fragment cloned into the *Hpa*I site (this study)
pVALAH	Kn^r (Mob$^+$ Rep$^-$) (Em^r)	pNJR-6 containing the 1.4-kb *Ava*I-*Hin*dIII fragment cloned into the *Hpa*I site (this study)

[a] Abbreviations for bacterial phenotypes: Str^r, streptomycin resistance; *melA7*, α-galactosidase minus (amber); Gen^r, gentamicin resistance. Abbreviations for plasmid phenotypes: Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance; Mob$^+$, can be mobilized; Rep$^+$, can replicate in *Bacteroides*; Rep$^-$, cannot replicate in *Bacteroides*. Plasmid phenotypes in parentheses are the phenotypes expressed in *Bacteroides*, whereas those not in parentheses are the phenotypes expressed in *E. coli*.

guar gum medium except that the heating step (70°C) was omitted. The final concentration of these polysaccharides was 0.5%. Melibiose, raffinose, and stachyose were filter sterilized and added to autoclaved defined medium to a final concentration of 0.5%. Glucose was added to the defined medium before it was autoclaved to a final concentration of 0.3%.

Cloning of the α-galactosidase gene. A cosmid library of *B. ovatus* chromosomal DNA was constructed, using pHC79 as the cosmid vector (6). Packaging extracts were prepared and reactions were done as described previously (3). Enzymes used in cloning procedures were purchased from Bethesda Research Laboratories (BRL Life Technologies, Inc., Gaithersburg, Md.) and used according to the manufacturer's specifications. *Sau*3A partial digests of the *B. ovatus* chromosome were size fractionated on an NaCl gradient (12). DNA fragments in the 30- to 40-kb size range were ligated with pHC79 which had been digested with *Bam*HI and treated with calf alkaline phosphatase (9). Packaged DNA was used to infect *E. coli* EM24, which was then plated onto LA medium containing ampicillin (75 μg/ml). *E. coli* EM24 strains containing individual cosmid clones were grown in LB medium and screened for α-galactosidase activity. Portions (2 ml) of each culture were pelleted by centrifugation and resuspended in 0.5 ml of potassium phosphate buffer (20 mM; pH 6.5) containing lysozyme (4 μg/ml). Resuspended bacteria were disrupted by rapid freeze-thaw lysis (three cycles).

Assays were done in microtiter dishes, using 100 μl of the disrupted cell preparations and 50 μl of 20 mM *p*-nitrophenyl-α-D-galactopyranoside (pNP-α-D-Gal) in potassium phosphate buffer (20 mM; pH 6.5). The microtiter dishes were incubated at 37°C for 2 h, and any color development was scored as positive. Under these conditions, *E. coli* EM24 did not exhibit any detectable α-galactosidase.

Transposon mutagenesis with Tn*1000* was done as described previously by Guthrie et al. (5). Transposon mutagenesis with the mini-Kan^r Tn*10* derivative was done by the method of Way et al. (25). Transformations were done as described by Lederberg and Cohen (11). Plasmids were isolated from *E. coli* and *Bacteroides* strains by the Ish-Horowitz modification of the method of Birnboim and Doly (12). Southern hybridization was done as described by Maniatis et al. (12).

Enzyme assays. α-Galactosidase activity in both *E. coli* and *Bacteroides* extracts was measured by determining the rate of hydrolysis of pNP-α-D-Gal at 37°C. The reaction mixture (0.5 ml) contained 0.4 ml of potassium phosphate buffer (20 mM; pH 7.0), 0.05 ml of 20 mM pNP-α-D-Gal, and 0.05 ml of appropriately diluted enzyme. The increase in A_{405} was measured with a Gilford recording spectrophotometer. A unit of enzyme activity was defined as 1 μmol of

p-nitrophenol liberated per min in potassium phosphate buffer (20 mM; pH 7.0) at 37°C. The extinction coefficient for *p*-nitrophenol under these assay conditions was 8.7×10^3.

Partial purification of the *B. ovatus* α-galactosidase from *E. coli*. *E. coli* EM24, containing plasmid pFG61ΔSE, was grown overnight in 3 liters of LB medium and harvested (10,000 × *g* for 10 min at 4°C). The pellet was washed two times with 100 ml of potassium phosphate buffer (20 mM; pH 7.0). The pellet was resuspended in the same buffer (40 ml), and cells were disrupted by passing them through a French pressure cell (12,000 lb/in^2). Cell debris was removed by centrifugation (17,000 × *g* for 20 min at 4°C). All subsequent steps were carried out at 4°C unless otherwise stated. The crude cell extract was centrifuged at 200,000 × *g* for 2.5 h. The membrane pellet was washed with potassium phosphate buffer (20 mM; pH 7.0) and centrifuged as before. The supernatants were combined and dialyzed against Tris hydrochloride buffer (20 mM; pH 8.5). The supernatants were applied to a DEAE-Sephacel column (2.5 by 20 cm) which had been equilibrated with Tris hydrochloride buffer (20 mM; pH 8.5). The column was washed extensively until no eluting protein was detected by A_{280}. The column was eluted with a linear 0 to 0.2 M NaCl gradient, and 3.5-ml fractions were collected. The fractions were assayed for enzyme activity, NaCl concentration, and protein (A_{280}). Fractions which contained enzyme activity were pooled. The yield was 60%, and there was a 20-fold increase in enzyme specific activity. The partially purified enzyme was stored at −20°C with 20% glycerol unless applied onto an isoelectric focusing (IEF) gel, in which case the enzyme preparation could not be frozen.

In a separate experiment, we estimated the native molecular weight of the α-galactosidase by using a Pharmacia FPLC (fast-protein liquid chromatography) Superose 12 column. The α-galactosidase [soluble extract of EM24 (pFG61ΔSE)] was dialyzed against 50 mM diethanolamine (pH 8.8) and applied to a mono-Q 5/5 anion-exchange column equilibrated with 50 mM diethanolamine (pH 8.8). Most of the activity eluted in a single fraction from the column at a sodium chloride concentration of 20%. A total of three fractions were pooled from three different experiments, and the pooled fractions were applied to a Superose 12 gel filtration column equilibrated with the same buffer plus 20% NaCl. The native molecular weight standards used were the following: thyroglobulin, 669,000; apoferritin, 443,000; β-amylase (sweet potato), 200,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 66,000; and carbonic anhydrase, 29,000.

Expression of the cloned gene in *Bacteroides* spp. To test for expression of the α-galactosidase gene in *Bacteroides*, the 8.8-kbp *Eco*RI fragment from pFG61 was cloned into the *E. coli-Bacteroides* shuttle vector pNJR-5 and mobilized from *E. coli* to *Bacteroides* as described previously (24). To construct gene disruption mutants, small fragments of the cloned DNA were cloned into the suicide vector pNJR-6, and the resulting plasmids were mobilized into *B. ovatus* with selection for erythromycin resistance (10 μg/ml).

To determine the α-galactosidase activity of these strains, the strains (100 ml) were grown to an optical density of 0.8 (A_{650}). Cells were harvested by centrifugation (10,000 × *g* for 10 min at 4°C). Cell pellets were washed with 50 ml of potassium phosphate buffer (20 mM; pH 7.0) and pelleted. The cells were resuspended in 5 ml of the same buffer and disrupted by using a Branson Sonifier (stepped microtip; output at 5 with pulsed setting; 50% duty) for two 3-min cycles. The α-galactosidase activity of these crude extracts was measured as described above. For IEF gel experiments, the crude extracts were separated into soluble and membrane fractions by an ultracentrifugation step. The crude cell extract was centrifuged at 200,000 × *g* for 2.5 h. The membrane pellet was washed with potassium phosphate buffer (20 mM; pH 7.0) and centrifuged as before. The supernatants were combined, and the membrane pellet was resuspended in 1.0 ml of the same buffer with 0.5% Triton X-100. In some experiments, similar extracts were prepared from *E. coli* by using the same procedure. Protein concentration was measured by the method of Bradford (1).

Substrate specificity. Cleavage of melibiose by partially purified α-galactosidase from *E. coli* EM24(pFG61ΔSE) and crude cell extracts of *B. ovatus* grown on melibiose or guar gum was assayed by the glucose oxidase assay and by descending paper chromatography. The amount of glucose released upon cleavage of melibiose was measured with the glucose oxidase kit (Sigma Chemical Co., St. Louis, Mo.) according to the manufacturer's direction. The solvent system used for descending paper chromatography was a solution of ethylacetate-acetic acid-water at a ratio of 3:1:1. α-Galactosidase (0.01 U) and 30 μg of substrate were mixed and incubated for 2 h at 37°C. The reaction was spotted onto Whatman no. 1 paper. Development and visualization of the chromatogram were done as described previously (4).

Cleavage of raffinose, stachyose, 4-*O*-α-D-galactopyranosyl-D-galactopyranose, intact guar gum, and partially degraded guar gum was also tested by descending chromatography. Partially degraded guar gum was prepared by incubating intact guar gum (5 mg/ml in 20 mM potassium phosphate, pH 6.5) with a membrane fraction from *B. ovatus* grown on guar gum for 30 to 180 min. Aliquots were taken at 30-min intervals and boiled for 5 min to stop the reaction. Carbohydrate (20 μg) from each time point was analyzed by decending paper chromatography. The latest time point that did not show any detectable galactose was used to test whether or not galactose residues could be cleaved from the guar gum fragments. Hydrolysis of *p*-nitrophenyl-glycosides other than pNP-α-D-Gal was tested as described above for pNP-α-D-Gal. All p-nitrophenyl glycosides were obtained by Sigma Chemical Co. Concentrations of the *p*-nitrophenol compounds were all 20 mM.

Maxicells and in vitro transcription-translation experiments. *E. coli* maxicell experiments were done as described previously by Sancar et al. (19). *E. coli* in vitro transcription-translation kits were purchased from Amersham Inc., and the procedure was done according to the manufacturer's directions except that the time allowed for both the [^{35}S]methionine labeling reaction and the cold chase was 30 min. [^{35}S]methionine was purchased from New England Nuclear Research Products, Boston, Mass.

Electrophoresis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (10) except that 50 mM iodoacetamide was added to the solubilizing solution. Proteins were visualized by staining with Coomassie brilliant blue. Prestained protein standards were purchased from Bethesda Research Laboratories, Gaithersburg, Md.

IEF was done with tube gels (5 mm by 13 cm). The samples were dialyzed against distilled H_2O prior to loading. Composition and electrophoresis of the gels have been described previously (4). After electrophoresis, gels were cut into 0.5-cm slices. For determinations of the isoelectric point, enzyme was eluted from the slices with 0.5 ml of potassium phosphate buffer (50 mM; pH 7.0). Slices from an identical gel, which contained IEF protein standards, were

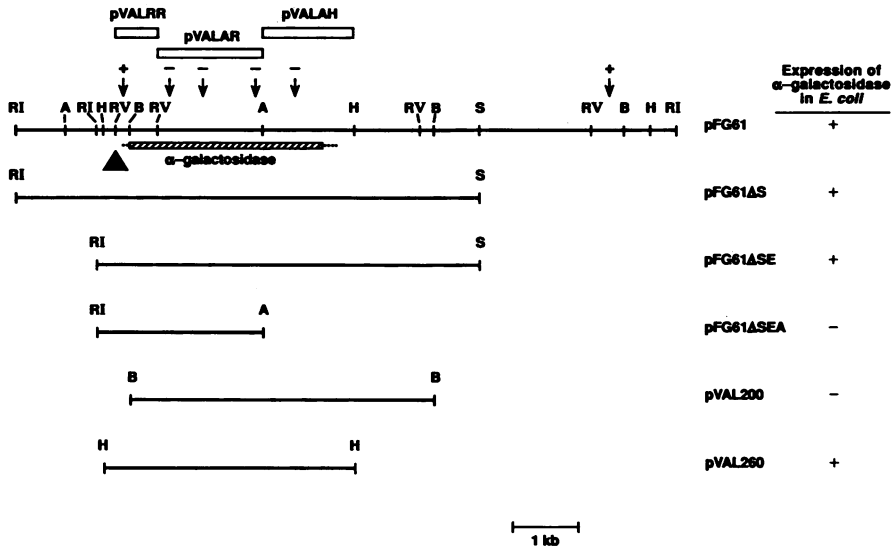

FIG. 1. Restriction map of subclones derived from cosmid clone p27-2. RI, *Eco*RI; H, *Hin*dIII; RV, *Eco*RV; B, *Bam*HI; A, *Ava*I; S, *Sph*I. The arrows above the first line denote transposon insertion sites, with a + or − signifying expression of the α-galactosidase gene in *E. coli*. The shaded rectangle below the first line indicates the position of the α-galactosidase gene, and the dots indicate that the exact ends of the gene are not known. The large solid triangle indicates the approximate location of the junction between two non-colinear fragments of DNA (see Results). The open boxes labeled pVALRR, pVALAR, and pVALAH signify the segments of DNA used to construct the chromosomal insertions in *B. ovatus* 0038.

eluted with distilled water and used to determine the pH gradient. Prestained IEF gel protein standards were purchased from Bio-Rad Laboratories, Richmond, Calif.

Germfree mouse experiments. BALB/c mice (male and female, 7 to 9 weeks old) were purchased from E. Balish, University of Wisconsin, Madison. Mice were maintained and fed as described previously (14, 26). Cultures of the wild-type and mutant *B. ovatus* strains were grown to an optical density of 0.6 to 0.8 (A_{650}) in TYG medium and combined to give a ratio of 0.1 or 0.4 of wild type to mutant. Mice were colonized by introducing a fresh mixed culture into the isolator and pouring culture on the food. A portion of the initial mixture was removed, and the ratio of wild type to mutant was determined. The mutant strain was distinguishable from the wild type because of the Em^r marker contained on the suicide vector which was inserted in the chromosome. At 2- to 5-day intervals, fecal pellets were collected, crushed with a sterile toothpick, and suspended in 0.5 ml of TYG broth. The fecal suspensions were diluted and plated, and then wild type were enumerated by picking 80 or 160 colonies onto TYG or TYG agar plus erythromycin (10 μg/ml). In some experiments, later time points were obtained up to day 60. When the ratio of wild type to mutant reached 80:1, we determined the ratio by plating the diluted fecal samples directly onto TYG agar and TYG agar plus erythromycin (10 μg/ml). Competition experiments were done with groups of three mice in each isolator, with each mouse in a separate cage. All experiments were repeated at least three times.

RESULTS

Cloning of the α-galactosidase gene. More than 300 cosmid clones were screened. One clone was found that expressed α-galactosidase activity in *E. coli*. This clone, designated p27-2, contained a 40-kb insert. p27-2 was partially digested with *Eco*RI and subcloned into pBR328. The smallest subclone which still expressed α-galactosidase activity in *E. coli* was a 10-kb *Eco*RI fragment designated pFG61 (Fig. 1). Sequential deletions using *Sph*I, *Eco*RI, and *Ava*I resulted in plasmids pFG61ΔS, pFG61ΔSE, and pFG61ΔSEA, respectively. Of these, only pFG61ΔSEA did not express α-galactosidase activity in *E. coli*. pVAL260, which contained the 3.6-kb *Hin*dIII fragment cloned into pBR328, also expressed α-galactosidase activity. However, pVAL200, which contained the 4.5-kb *Bam*HI fragment cloned into the *Bam*HI site of pNJR-5, was inactive. Thus, one end of the gene lies between the *Hin*dIII and *Bam*HI sites, and the other lies between the *Hin*dIII and the *Ava*I sites. The location of the gene was further confirmed by transposon mutagenesis, using Tn*1000* and a mini-Tn*10* kanamycin derivative (Fig. 1).

To determine whether the restriction pattern of the region containing the cloned α-galactosidase gene was the same as the chromosomal locus in *B. ovatus*, we probed *Eco*RI-digested chromosomal DNA with pFG61ΔS. The results indicated that the cloned DNA was not colinear to the *B. ovatus* chromosome (data not shown). The sizes of the *Eco*RI bands predicted from the restriction map of pFG61 were 1.2 and 8.8 kb. However, the Southern blot of chromosomal DNA had three cross-hybridizing *Eco*RI bands: 1.2, 6, and 16 kb. When *Eco*RI-digested *B. ovatus* chromosomal DNA was probed with pVAL260, pVALAR, or pVALAH, a single 16-kb *Eco*RI band was observed in all three cases. Also, the size of the *Hin*dIII band predicted from the restriction map of pFG61 when pVAL260, pVALAR, or pVALAH was used as the probe was 3.7 kb. However, a 6.0-kb band cross-hybridized with each probe. Thus, since the size of the original cosmid clone (p27-2) indicated that a deletion had not occurred after infection of the *E. coli* host, there may have been two different *Sau*3A chromosomal fragments joined prior to packaging in lambda extracts. The junction of the two segments of DNA was found to be to the left side of the 3.7-kb *Hin*dIII fragment. This was evidenced by the fact that the 4-kb *Eco*RV frag-

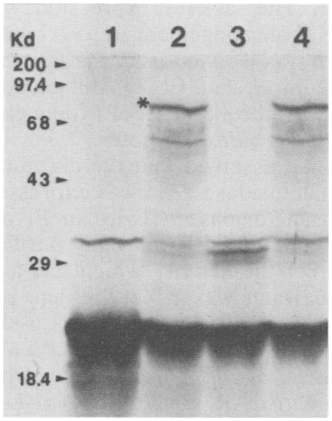

FIG. 2. Autoradiogram of the [^{35}S]methionine-labeled protein products from an in vitro coupled transcription-translation experiment. Templates used in the experiment were as follows: lane 1, pBR328; lane 2, pVAL260 with a mini-Tn*10* insertion in the ampicillin gene; lane 3, pVAL260 with a mini-Tn*10* insertion in the α-galactosidase gene; lane 4, pVAL260. The 78-kDa protein product is marked with an asterisk. The molecular weights of the antibiotic resistances encoded by pBR328 are the following: Tc^r, 36,000; Ap^r, 28,000; Cm^r, 23,000. Neither the Tc^r nor the Kn^r gene product encoded by the Mini-Tn*10* was seen on autoradiograms of in vitro transcription-translation experiments. The sizes of protein standards are shown.

ment that overlaps the right side of the 3.7-kb *Hin*dIII but not the 3-kb *Ava*I fragment which overlaps the left end of the *Hin*dIII fragment was seen when pFG61ΔS was used as a probe (data not shown). However, the coding region of the cloned α-galactosidase gene and everything else to the right in Fig. 1 were still intact. At no point in the Southern analysis did we see evidence for a second locus that hybridized with the cloned region.

Biochemical characterization of the partially purified α-galactosidase from *E. coli* extracts. The pH optimum for the enzyme was 6.7 to 7.2. There was no enhancement of α-galactosidase activity when Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, or NADH was included in the reaction mixture. The pI of the enzyme was 7.6, as compared with the pI values of 5.6 and 6.9 obtained previously for α-galactosidases I and II, respectively. When a mixture of the partially purified enzyme and a soluble extract of *B. ovatus* grown on melibiose was applied to one IEF gel, α-galactosidase II was well separated from the peak which corresponded to the cloned enzyme (data not shown).

When pVAL260 was used as template in an in vitro transcription-translation system, a 78-kDa protein was seen that was not seen with the vector-minus insert or with a transposon insertion that eliminated α-galactosidase activity (Fig. 2). The 78,000-Da protein was also observed in extracts of maxicells carrying pVAL260 (data not shown). A minor protein of 60,000 Da was associated with the cloned region in the in vitro assays, but this protein was probably an artifact of the in vitro system because it was not seen in maxicell extracts. Thus, the 78,000-Da protein appears to be the α-galactosidase gene product. The monomeric molecular weights of α-galactosidases I and II had been previously determined to be 85,000 and 80,500, respectively (4). The native molecular weight of the α-galactosidase, when analyzed by FPLC gel filtration with a Superose 12 column, was determined to be 150,000 ± 15,000. Thus, the α-galactosidase appears to function as a dimer.

Under optimum conditions, melibiose was the only α-galactosidase other than pNP-α-D-Gal which was cleaved by the partially purified enzyme. By contrast, α-galactosidases I and II not only hydrolyzed pNP-α-D-Gal and melibiose, but also were able to remove galactose residues from raffinose, stachyose, and partially degraded guar gum. None of the three enzymes was able to degrade the disaccharide 4-*O*-α-D-galactopyranosyl-D-galactopyranose.

The rate at which the partially purified α-galactosidase cleaved melibiose was lower than that of α-galactosidase II. When the substrate concentration was in excess (10 mM), the partially purified α-galactosidase was 56% as active as α-galactosidase II when equal amounts of units (based on pNP-α-D-Gal assay) were used in the glucose oxidase assay with melibiose as the substrate. Given the relatively low activity of the enzyme against melibiose and its lack of activity against any of the other α-galactosidases, we considered the possibility that pNP-α-D-Gal and melibiose might not be the primary substrates. However, under conditions which optimized activity on pNP-α-D-Gal, the α-galactosidase had no detectable activity on any of the following *p*-nitrophenol (pNP) substrates: pNP-α-D-arabinopyranoside, pNP-α-D-fucoside, pNP-α (or β)-D-mannopyranoside, pNP-α (or β)-D-glucopyranoside, pNP-β-D-melibiose, pNP-β-D-galactopyranoside, oNP or pNP-*N*-acetyl-α-D-galactosaminide, pNP-*N*-acetyl-α-D-glucosaminide, pNP-2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranoside, pNP-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside, and pNP-2-acetamido-2-deoxy-3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-galactopyranoside.

Expression of the gene in *B. ovatus*. Since the biochemical characteristics of the *B. ovatus* α-galactosidase gene expressed in *E. coli* differed from those of α-galactosidases I and II, it appeared that the cloned enzyme was a third α-galactosidase. A previous study in which α-galactosidase activities of *B. ovatus* were purified had not yielded any evidence of a third α-galactosidase (4). However, an enzyme expressed at low levels might have been missed in the purification. Alternatively, simple α-galactosidases and galactomannans such as guar gum may not be natural inducers of this α-galactosidase. Since the pI of the α-galactosidase expressed in EM24(pFG61ΔSE) was different from the pI of α-galactosidase I or II, it should be possible to detect it on IEF gels. Accordingly, we determined the α-galactosidase pattern in IEF gels of soluble and membrane fractions of *B. ovatus* cells which had been grown on glucose, mannose, melibiose, guar gum, xylan, arabinogalactan, pectin, polygalacturonic acid, gum tragacanth, porcine mucin, or trypsin inhibitor.

An α-galactosidase activity which migrated at the pH (7.6) predicted for the enzyme expressed in EM24(pFG61ΔSE) was found in extracts of *B. ovatus* that was grown on most of the carbon sources tested. The α-galactosidase pattern observed when a soluble extract of glucose-grown *B. ovatus* is applied to a gel is shown in Fig. 3. The three peaks of activity are well separated, and the level of activity in the peak corresponding to the cloned gene (pI 7.6) was very low. The activity which focused at pH 7.6 was seen only in the soluble fraction of cell extracts and not in the membrane fraction. In contrast, both α-galactosidases I and II could be detected in membrane and soluble fractions, although most of the activity was soluble. There was no significant difference in the levels of the pI 7.6 peak between glucose-grown cells and

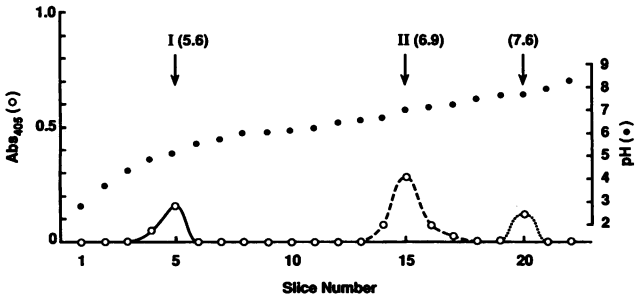

FIG. 3. Separation on an IEF gel of α-galactosidase I (–○–○–; pI 5.6), α-galactosidase II (○- -○- - -; pI 6.9), and a third α-galactosidase (· · ·; pI 7.6) in soluble extracts of *B. ovatus* which were grown on glucose. A total of 100 μg of protein was loaded on the gel. The eluted proteins from the slices containing α-galactosidase activity were incubated with pNP-α-D-Gal for 2.5 h, and the absorbance was measured.

cells grown on most of the other substrates except for gum tragacanth and porcine mucin (Table 2).

Soluble extracts of *B. ovatus* which had been grown on gum tragacanth and porcine gastric mucin contained an additional α-galactosidase activity (α-galactosidase IV) which was not detected under other growth conditions (Table 2). The α-galactosidase activity had a pI of 4.5. No further characterization of this enzyme was done.

Testing expression of the cloned α-galactosidase gene product in *B. ovatus*. Introduction of the cloned α-galactosidase gene into *B. ovatus* on a multiple-copy shuttle vector should result in a detectable increase of activity having a pI of 7.6, since the background level was shown to be very low. We first tried cloning the 8.8-kb *Eco*RI fragment containing the α-galactosidase gene into pNJR-5 to construct pVAL250. This plasmid was mobilized into *B. ovatus*. The level of α-galactosidase activity in extracts from *B. ovatus* containing pVAL250 was identical to the level in *B. ovatus* containing no plasmid. The lack of expression was not due to mutation or DNA rearrangement in pVAL250 because plasmid DNA which was isolated from the *B. ovatus* strain, and introduced back into *E. coli* EM24, still encoded α-galactosidase activity. Thus, the 8.8-kb *Eco*RI fragment may not have contained the *Bacteroides* promoter.

TABLE 2. α-Galactosidase activities in soluble extracts detected on IEF gels when *B. ovatus* is grown on various carbohydrates

Carbohydrate source[a]	Relative levels[b] of α-galactosidase detected			
	pI 4.5 (IV)[c]	pI 5.6 (I)	pI 6.9 (II)	pI 7.6 (V)
Glucose/mannose	<0.02	1	1	1
Melibiose	<0.02	1	14	1
Guar gum	<0.02	20	2	1
Arabinogalactan	<0.02	1	4	1
Xylan	<0.02	1	3	1
Polygalacturonic A	<0.02	1	2	1
Trypsin inhibitor	<0.02	1	4	1
Pectin	<0.02	2	1	1
Mucin (porcine)	1[d]	3	2	<0.1
Gum tragacanth	5	<0.05	<0.03	<0.1

[a] Defined medium supplemented with the above carbohydrates. See Materials and Methods for further explanation.

[b] The level of α-galactosidase activity from glucose- or mannose-grown cells was taken as 1 for each peak of activity on an IEF gel. The levels of α-galactosidase from cells grown on other carbohydrates were given values corresponding to the fold increase in the level of expression of each activity. The limit of detection was 0.01 U/ml.

[c] Roman numerals indicate the different α-galactosidases.

[d] For the α-galactosidase having a pI of 4.5, the amount detected could not be compared with glucose/mannose-grown conditions. Therefore, the level of activity for mucin-grown cells was taken as 1.

Initially, we had assumed that the chromosomal copy of the cloned α-galactosidase gene was expressed in *B. ovatus* because an α-galactosidase activity in *B. ovatus* extracts focused at the same pI as the partially purified enzyme. To determine whether the activity which had a pI of 7.6 in *B. ovatus*-soluble extracts was in fact the same protein encoded by the clone in *E. coli*, we used the 1.6-kb *Ava*I-*Eco*RV fragment (pVALAR, Fig. 1) to construct a single crossover insertion in the *B. ovatus* chromosome. This mutant was designated *B. ovatus* AR. Since the *Ava*I-*Eco*RV fragment was internal to the cloned gene, the insertion in *B. ovatus* AR should disrupt the gene in the chromosome. The pattern of α-galactosidase activity obtained with soluble extracts from the disruption mutant was identical to that of the wild type. In particular, the peak of activity at pH 7.6 was still present in the mutant. Thus, the enzyme in *B. ovatus*-soluble extracts which focused at a pH of 7.6 appeared to be distinct from the partially purified α-galactosidase as well as from α-galactosidases I, II, and IV. We designated the α-galactosidase encoded by the cloned gene α-galactosidase III and the enzyme detected in extracts of *B. ovatus* at pI 7.6 α-galactosidase V. These results also confirm that we did not clone α-galactosidase I or II since there was no change in the level of activity in the region of the gels which corresponded to their respective pI values of 5.6 and 6.9.

Competition experiments. The growth rate of *B. ovatus* AR grown on defined medium plus glucose, melibiose, raffinose, or guar gum was the same as that of the wild-type strain. Moreover, when *B. ovatus* AR and wild type were mixed and inoculated into the same tube of medium, the ratio of wild type to mutant remained constant after eight transfers. Thus, α-galactosidase III appeared not to be important for growth on any of the substrates tested. Nonetheless, it appeared to be important for colonization of the gastrointestinal tract of germfree mice. The results of putting *B. ovatus* AR in competition with wild type in germfree mice are shown in Fig. 4A. The mutant was outcompeted by the wild type strain. The ratio of wild type to mutant reached 10^4:1 within 30 days in two experiments and 10^6:1 within 60 days for the third experiment after introducing the mixed culture into the mice.

The disruption in α-galactosidase III could have had an effect in vivo because of a polar effect on some downstream gene. To ascertain whether this was the case, we made disruptions on either side of α-galactosidase III. The DNA fragments used to make the insertions were the 0.6-kb *Eco*RV fragment (pVALRR, Fig. 1) and the 1.4-kb *Ava*I-*Hin*d III fragment (pVALAH, Fig. 1). The *B. ovatus* strains containing the insertions were designated *B. ovatus* RR and AH, respectively. All three mutant strains, RR, AR, and AH, were confirmed to have the insertions within the 6.0-kb *Hin*dIII fragment as predicted by the Southern blot data. *B. ovatus* RR and AH were both outcompeted by wild type at a rate similar to that seen with *B. ovatus* AR (Fig. 4B).

To rule out the possibility that the deleterious effect of the mutation was caused by the exogenous DNA used to generate the insertion, we needed to test another strain of *B. ovatus* which contained the same exogenous DNA inserted elsewhere in the chromosome. *B. ovatus* X1, which was constructed in the same fashion as *B. ovatus* AR, RR, and

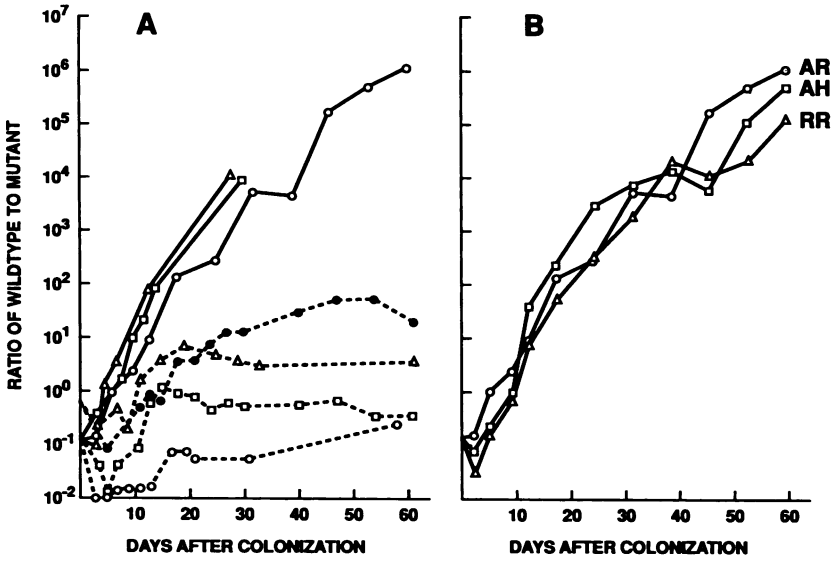

FIG. 4. Results of long-term competition experiments in germfree mice. Each point represents the ratio of wild type to mutant from three mice, and the value at each time point is the arithmetic mean of the three mice. (A) Data from the following competition experiments: *B. ovatus* AR versus wild type (solid lines) and *B. ovatus* X1 versus wild type (dashed lines). In two of the experiments involving *B. ovatus* X1, the initial ratio was 0.4 at day 0 (closed circles and triangles), and in the other two experiments the ratio was 0.1 (open circles and boxes). (B) Data from the following competition experiments: wild type versus *B. ovatus* RR (triangle), wild type versus *B. ovatus* AR (open circle), and wild type versus *B. ovatus* AH (open box). The results obtained with *B. ovatus* X1 were more variable than those obtained with the other mutants. Thus, all of the *B. ovatus* X1 competition experiments are shown to give an idea of the range of values. Only one experiment is shown for each of the other mutants because the results from replicate experiments were virtually identical to those shown here.

AH but in which a different segment of target DNA was used, was able to compete with the wild-type strain (Fig. 4A). There was considerable variation in the *B. ovatus* X1-versus-wild type competition experiments, and the final ratio of wild type to mutant depended to some extent on the ratio used to colonize the animals. However, in all cases the ratio of wild type to X1 leveled off at a value of ≤50. By contrast, in competition experiments which involved *B. ovatus* AR, RR, and AH, the ratio of wild type to mutant continued to increase throughout the sampling period.

DISCUSSION

α-Galactosidase III differed from the two previously characterized enzymes from *B. ovatus* with respect to its pI and molecular weight and in its substrate spectrum. Because α-galactosidase III was able to degrade only melibiose but not raffinose, stachyose, or partially degraded guar gum, it would seem that it might play a role in the utilization of melibiose. However, aside from α-galactosidase II, melibiose did not induce an α-galactosidase activity in *B. ovatus* which resembled α-galactosidase III. Also, disruption of the α-galactosidase III gene did not affect growth of *B. ovatus* on melibiose or any other substrate we tested. One explanation would be that the *Bacteroides* DNA, when in *E. coli*, derepressed an *E. coli* α-galactosidase. However, the native molecular weight observed for α-galactosidase III (150,000 ± 15,000) was consistent with the monomeric molecular weight seen in the in vitro transcription translation assays (78,000); therefore, it appears that the 78-kDa polypeptide was the α-galactosidase.

Although α-galactosidase III was expressed in *E. coli* as a functional enzyme, indicating that there is an intact open reading frame in the clone, it is not clear whether the gene is expressed in *B. ovatus*. A low but detectable level of α-galactosidase activity focused in IEF gels at the same pH as α-galactosidase III, but this was not the same as the enzyme which was cloned in *E. coli*, because it was still present in the disruption mutant, *B. ovatus* AR. This result, together with the lack of increase in α-galactosidase activity when the cloned gene was introduced into *B. ovatus* on a multicopy plasmid, could indicate that the gene is cryptic in *B. ovatus*. A more likely explanation is that α-galactosidase III is an inducible enzyme in *B. ovatus* and none of the substrates we tested was the inducer. For example, expression of α-galactosidase IV would not have been detected at all if porcine gastric mucin and gum tragacanth had not been tested. The ability to degrade melibiose does not preclude other carbohydrates from being the natural substrate of α-galactosidase III. We tested a number of polysaccharides which contain some proportion of α-linked galactose residues, but did not find any which induced α-galactosidase III. Although many different α-galactoside-containing polysaccharides are found in nature, many of these are not commercially available. Thus, the real inducer may not be available for testing.

Evidence that α-galactosidase III may be expressed in vivo by *B. ovatus* came from experiments which showed that the gene disruption adversely affected competition with wild type for colonization of the gastrointestinal tracts of germfree mice. The mice were fed a complex chow diet which could contain a carbohydrate component that acts as the inducer of the cloned α-galactosidase gene. Since both of the flanking insertions in the *B. ovatus* chromosome had the same deleterious effect in vivo, other genes in this region also appear to play an important physiological role. This is not surprising in view of the fact that multiple genes are normally required for utilization of a complex carbohydrate.

It was possible that the effect of the insertion mutations on colonization was not directly due to disruption of the chromosomal locus containing α-galactosidase III but to the

introduction of exogenous DNA. Previous experiments involving a closely related species, *B. thetaiotaomicron*, had indicated that introducing plasmid DNA into the chromosome had no effect on competition in vivo. However, this result might not apply to *B. ovatus*. Presently, only four insertion mutants (*B. ovatus* RR, AR, AH, and X1) have been constructed in *B. ovatus* 0038. The same suicide vector, pNJR-6, was used in all cases. Thus, *B. ovatus* X1 was the only strain which could be used to test the effect of insertion of the exogenous DNA. The X1 insertion had a slightly deleterious effect because the ratio of wild type to mutant increased after the initial introduction of the mixture into the germfree mice. However, the ratio of wild type to *B. ovatus* X1 never exceeded 50 as it did in the *B. ovatus* RR, AR, and AH competition experiments. *B. ovatus* X1 has not been as well characterized as the other three insertion mutants. The insertion in *B. ovatus* X1 has no detectable effect on growth in laboratory media, but this does not prove that it is a completely neutral mutation. Recently, Western blot (immunoblot) analysis of the membrane protein profile of *B. ovatus* X1 has indicated that this mutant is missing a constitutive membrane polypeptide (data not shown). The loss of the polypeptide rather than the exogenous DNA introduced into the mutant strain could explain why the insertion in *B. ovatus* X1 had a slight effect in vivo. In any event, the insertions in the α-galactosidase locus were clearly more deleterious than the insertion in *B. ovatus* X1.

Some possible physiological roles for the α-galactosidase other than carbohydrate catabolism have been considered. For example, the enzyme could be involved in a transferase type reaction involving the transfer of galactose to a growing chain of carbohydrate destined for lipopolysaccharide. However, there was no evidence of band shift or changes in the spacing of the lipopolysaccharide ladder (unpublished data). Also, there was no change in colony morphology or in the susceptibility to antibiotics. Thus, the genes in this chromosomal locus appear to be important for competitiveness in vivo, but their role in the cell is still unclear.

In the process of characterizing the insertion mutant *B. ovatus* AR and searching for an inducer of α-galactosidase III, we detected two additional α-galactosidases. Expression of one of them, α-galactosidase IV, was associated with growth on porcine gastric mucin and gum tragacanth. This was somewhat surprising since α-galactosidase residues are thought to be a minor component of these polysaccharides. The finding that a specific enzyme is induced by *B. ovatus* when polysaccharides which have a small proportion of α-galactoside linkages are utilized indicates that *B. ovatus* is highly adapted to utilization of galactosides from any available source. The fifth α-galactosidase, which had the same pI as α-galactosidase III, appeared to be regulated because it was not detected in bacteria grown on porcine gastric mucin and gum tragacanth. However, none of the substrates tested produced elevated levels of this enzyme. In this case, as with α-galactosidase III, the actual inducer may not be commercially available. It is often assumed that any gene that is important in vivo should have an easily detectable activity or phenotype when cells are grown in laboratory medium. Our results indicate that this assumption is not always correct and that there are some complex carbohydrates which are important in the colon but which are not commercially available.

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